Neutral red as an indicator of pH transients
in the lumen of thylakoids – some answers to criticism

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In thylakoids light-driven proton pumps operate between the chloroplast stroma and the very narrow lumen. Under selective buffering of the suspending medium small flash-induced transients of the surface pH at the lumenal side of thylakoids are measurable via absorption changes of neutral red. The indicator acts specifically and quantitative (Junge, W., Ausländer, W., McGeer, A. and Runge, T. (1979) Biochim. Biophys. Acta 546, 121–141 and Hong, Y.Q. and Junge, W. (1983) Biochim. Biophys. Acta 722, 197–208). Recently, this was challenged by De Wolf et al. (De Wolf, F.A., Groen, B.H., Van Houte, L.P.A., Peters, F.A.L.J., Krab, K. and Kraayenhof, R. (1985) Biochim. Biophys. Acta 809, 204–214) by claiming these absorption changes indicated "charge rearrangements at the thylakoid exterior domains". This view is not compatible with the cited studies on the response mechanism of this dye in thylakoids. Moreover, its implications are inconsistent with accepted features of photosynthesis in thylakoids. We determined the redistribution time of neutral red in response to a small pH difference across the membrane. This was rather long (10 s). It explained why the dye was well suited as a quantitative indicator of small pH transients at short time intervals, but less well suited at longer time scales. We measured the passage of protons across thylakoid membranes as viewed from the lumen (via neutral red) and as viewed from the medium (via phenol red). Both types of measurement revealed the same rates of proton flow under variation of the rate over three orders of magnitude. Hence, protons were completely tracked on their way from the lumen into the medium.

Introduction

At the thylakoid membrane, proton flux is measurable at high time resolution in three different ways, via pH transients in both aqueous compartments and, in addition, via voltage transients across the membrane. This is due to the availability of spectrophotometric measuring techniques for (a) the transmembrane voltage (Ref. 1; for reviews, see Refs. 2 and 3); (b) pH transients in the medium [31,35]; and (c) pH transients in the lumen (Ref. 4, reviewed in Ref. 5). The latter technique relies on neutral red as a membrane adsorbed pH indicator. Two examples illustrate its usefulness. (a) Photosynthetic water oxidation occurs at the lumenal side of the thylakoid membrane (see Ref. 6 for a recent review). Via neutral red the four sequential steps of water oxidation, as accompanied by proton liberation, were studied [7–10]. This led to a convergent scheme for the

pattern of proton release in this four-step catalytic process which agreed with the one which resulted from studies with pH electrodes, via fluorescence emission, via magnetic resonance (for references, see Ref. 12) and with a pH-indicating spin probe [11]. The neutral red technique, however, was superior for time resolving the partial reactions of proton release [12–14]. (b) The neutral red technique was also directed towards photophosphorylation and in particular to the highly debated existence of localized (or intramembrane) pathways for protons from pumps into ATP synthase molecules. Protons, liberated during water oxidation, under certain conditions were transiently trapped in 'intramembrane domains' [15–17]. This finding paralleled the observation that the lag for the onset of photophosphorylation under excitation with a series of light flashes was prolonged [18]. Neutral red revealed that transient trapping ended after a limited pool had been filled with protons derived from water oxidation (see Ref. 17).

Work with spectroscopic probes for electrochemical events has led to several pitfalls, which are documented in the early bioenergetic literature. The use of neutral red as described by Ausländer and Junge [4] was criticized by Pick and Avron [19], and one warning on the same line emerged from earlier work on submitochondrial particles by Dell'Antone et al. [20,21]. These authors did not find the spectroscopic response of this dye straightforwardly related to the relatively large pH transients, which were induced by prolonged operation of proton pumps. We have always agreed with the notion that redistribution of the dye between water and membrane and subsequent dimerization of the bound dye does not recommend its application as a quantitative indicator for large pH transients at longer time scales (above some seconds). On the other hand, we investigated in detail the physico-chemical mechanism of its response to small (in the order of 0.01 pH units) and rapid (less than 1 s) pH transients which are induced in thylakoids by single-turnover flashes of light [22,23]. The following emerged from these studies. (a) neutral red was absorbed to both sides of the thylakoid membrane; (b) it was capable of detecting pH transients in the lumen by appropriate buffering of the medium; (c) it acted

as a true indicator of the interfacial pH (i.e., its apparent pK was modulated by the surface potential); (d) artefacts associated with possible redox reactions of neutral red were absent.

Recently, De Wolf et al. [24] challenged this interpretation of the absorption changes of neutral red in thylakoids. According to these authors they instead "...may as well reflect electric-charge rearrangements at the thylakoid exterior domains...in many respects similar to...the aminoacridine probe". This alternative view was based on extrapolation of studies with neutral red in Photosystem I particles, but not on an extension or correction of our experiments on thylakoids or of their theoretical evaluation. We do not attempt to argue about the validity of their interpretation for Photosystem I particles, but there are two obvious arguments which eliminate this interpretation at least for thylakoids. (1) If neutral red was to "reflect electric-charge rearrangements" in thylakoids, then it is extremely difficult to understand why its response was quenched by various, chemically very different buffers (see Fig. 3 in Ref. 22), but not by relatively high concentrations of Mg^{2+} (20 mM). Mg^{2+} is a potent quencher of surface potentials and, moreover, its activity in thylakoids at the site of neutral red-adsorption was apparent from the Mg^{2+}-induced pK shift of the adsorbed neutral red (see Figs. 1 and 3 in Ref. 23). (It is noteworthy, that De Wolf et al. [24] failed to document the influence of Mg^{2+} on the apparent pK of neutral red in their research object.) (2) If the absorption changes of neutral red were responding to events at "the thylakoid exterior domains" then their above-cited kinetic reflection of the characteristically four-step progress of water oxidation is difficult to understand. It either implied that the catalyst for water oxidation was located in thylakoid exterior domains or, at least, that it released protons into such exterior domains. This proposal is conflicting not only with the above-cited work on the sidedness of proton release by water oxidation (both via neutral red and via a spin probe), but also within an independent body of evidence for lumenal location of water oxidation resulting from various biochemical and biophysical approaches in different laboratories (for reviews, see Refs. 6, 25 and 27).

Therefore, the extrapolation by De Wolf et al.

(1985) of their work on Photosystem I-particles to thylakoids is not only methodologically questionable, but their hypothesis concerning the response mechanism of neutral red in thylakoids is most probably wrong. Nevertheless, we took the criticism by De Wolf et al. [24] as an opportunity to present further experiments on this method in order to improve the understanding of the limits of applicability. Furthermore, we demonstrated complete tracking of protons on their way from the lumen across the membrane into the medium.

We include a brief review of former results to which the new experiments are directly related.

Materials and Methods

Thylakoids were prepared from peas according to the procedure for "stacked chloroplasts" in Ref. 28. EDTA-treated thylakoids (Fig. 3) were also prepared according to Ref. 28. Aliquots from the concentrated stock suspension (3–5 mM chlorophyll/ml) showed no effects of aging when used for up to 5 h after preparation. Thylakoids used for one experiment shown in fig. 1 (middle trace) were prepared as in Ref. 12, suspended in a medium containing DMSO (5%) as cryoprotective, frozen under liquid nitrogen and stored until use. For photometric experiments aliquots taken from the respective stock suspension were suspended at 10 or 20 μM chlorophyll in unbuffered media, except for thoses cases where bovine serum albumin was added (see figure legends). The pH was adjusted by addition of HCl or NaOH and it was stable within $\pm$ 0.1 units during measurement. All experiments were at room temperature.

Absorption changes were measured in a flash spectrophotometer as described in Refs. 10 and 30. The path-length of the absorption cell was 20 mm. "pH_{out}-indicating absorption changes of phenol red" are the difference between a transient signal obtained in the presence and another one obtained in the absence of this dye [31]. "pH_{in}-indicating absorption changes of neutral red" are also the difference between a transient signal measured with and one measured without addition of the dye. They were obtained in the presence of bovine serum albumin (2.6 mg/ml) as selective buffer for the suspending medium [22]. The measuring wavelengths were 548 nm (neutral red) and

559nm (phenol red), respectively. pH indicator dyes were added to the suspension at a concentration of 15 μM.

Results and Discussion

Neutral red is a lipophilic pH indicator with an electroneutral base form and with a pK in water of 6.6 (Refs. 4, 23 and 24 – see these articles for references to other pK values from the literature). In the thylakoid suspension with neutral red flash excitation produces additional absorption changes in the wavelength region around 520 nm. These were tentatively attributed to an acidification of the thylakoid lumen [32]. Closer inspection showed that they were composite from a negatively directed transient ('alkalinization') and a positively directed one ('acidification') [4]. The negatively directed component was fully quenched by addition of buffers including the certainly non-permeant bovine serum albumin, while the positive directed component was sensitive only to smaller buffer molecules. This led to the notion that neutral red responded to pH transients at both sides of thylakoid membranes and that the response to transients at the lumenal side could be obtained under selective buffering of the outer medium by bovine serum albumin [4]. The pH_{in}-indicating absorption changes of neutral red resulted from measurements with suspensions buffered by bovine serum albumin (more than 1.3 mg/ml) by subtraction of transients measured in the absence from those in the presence of neutral red. The quantitative nature of this indicator has been established in two manuscripts [22,23]. The following three properties emerged from these studies.

(1) Neutral red was adsorbed to the surface of thylakoid membranes to a degree which depended on the medium pH and on the surface potential. If the latter was screened by magnesium cations (20 mM) the adsorption of neutral red was pH independent. This implied that the pK of neutral red in the neutralized membrane was he same as in water, namely 6.6. The distribution coefficient of neutral red between a neutralized membrane and water was $c(NR_m)/c(NR_{water}) = 880$ (the figure of 285 000 given in Ref. 23 is erroneous). Neutral red binding as well as the pH and the salt dependence of its transient response behaved as if its

apparent pK depended on the surface potential, φ, as follows:

$$pK(\varphi) = 6.6 - \frac{F}{2.3\,RT}\,\varphi$$

Hence neutral red acted as an indicator of the surface pH.

(2) Because of this equation its transient response to activation of the proton pumps resulted from two components: the actual pH transient, and, via the pK shift, the transient of the surface potential caused by the former. We calculated the electric portion of the response which was negligible even at relatively low concentration (10 mM) of a uni-univalent electrolyte (see p. 207 in Ref. 23). The most direct argument in favour of a dominant pH response came from the effect on these absorption changes of various buffers which greatly differed in their chemical properties. These buffers, including hydrophilic ones like PP$_i$, quenched the transient response of neutral red to a degree which depended in the expected way on their buffering capacity in aqueous solution. Even more convincingly, these buffers quenched more efficiently the larger the internal volume of thylakoids [22].

(3) The absence of other responses which might be caused by, e.g., the reduction of neutral red was demonstrated (see fig. 2 in Ref. 23). The pH transients in the lumen which were induced by excitation with single-turnover flashes were calibrated (0.06 pH units) (Ref. 22; note that 0.6 in the penultimate line of its Summary is erroneous). The same magnitude was corroborated in another laboratory by EPR [11].

The above interpretation of neutral red acting as an indicator of the surface pH at the lumenal side of thylakoid membranes relied on the pronounced effects of buffers and of cations on its absorption changes. The observed effect of, e.g., phosphate buffer on these absorption changes has caused discussion. Hangarter and Ort [33] observed that hydrophilic buffers like phosphate were not taken up in the thylakoid lumen. On the same line, we found no influence of phosphate and of cations on the pH$_{in}$-indicating absorption changes of neutral red if we worked with thylakoids which were used fresh after preparation as contrasted to those which were used after freezing, storage un-

der liquid nitrogen and thawing [23]. Thus there were two classes of thylakoids: one ('frozen') sensitive to added buffers and cations where the pH-indicating properties of neutral red could be established, and another one ('fresh') where the obvious tests via buffers and cations did not work. The following experiment gives evidence that neutral red operated the same way in both afore-mentioned types of thylakoid.

The kinetic properties of the absorption changes of neutral red in freshly prepared thylakoids with the lumen not accessible to hydrophilic buffers
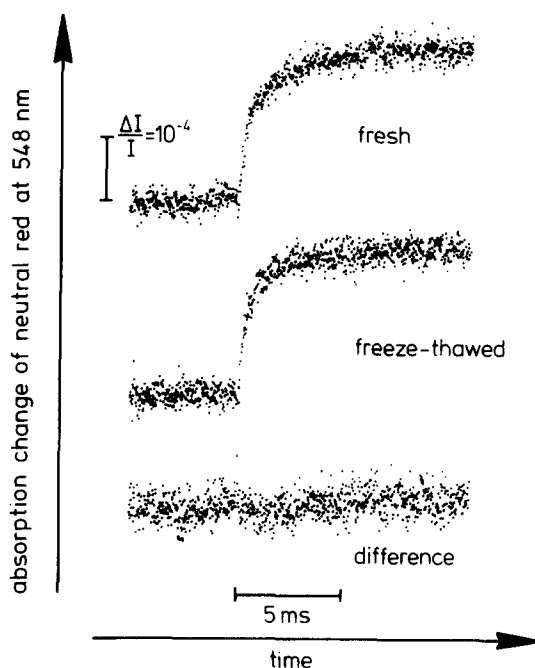
Fig. 1 shows the pH$_{in}$-indicating absorption

Fig. 1. Flash-induced absorption changes of neutral red (+bovine serum albumin ± neutral red) under repetitive excitation (flash frequency, 0.1 Hz). Thylakoids at a chlorophyll-concentration of 10 μM were suspended in a medium with hexacyanoferrate(III) (2 mM), as electron acceptor to Photosystem II, DBMIB (10 μM) added to block electron transfer via the cytochrome b_6/f complex (under these conditions water oxidation was the only protolytic reaction site [12]), KCl (25 mM) and MgCl$_2$ (3 mM). pH 7.5 (see Figs. 3 and 5 in Ref. 23 for the influence of salt and pH on the absorption changes of neutral red). Top: freshly prepared thylakoids; middle: freeze-thawed thylakoids which were stored under liquid nitrogen under cryoprotection by DMSO (5%); bottom: difference between the two upper traces.

changes of neutral red in two types of thylakoid, freshly prepared (upper trace) and freeze-thawed (middle trace). The difference between these two transients vanished (bottom trace). Thus the absorption changes of neutral red were identical, in extent as in kinetic behaviour, which showed that neutral red reacted the same way in fresh chloroplasts as in freeze-thawed ones, namely as indicator of the lumenal surface pH.

Neutral red is a very mild protonophore

Fig. 2 shows the pH_{out}-indicating absorption changes of phenol red and the pH_{in}-indicating absorption changes of neutral red. The positively directed absorption changes of phenol red reflected the alkalization of the medium. Their rise kinetics were complex and with half-rise times in the order of 100 ms (by the delayed diffusion of protons through the buffering partition domains of stacked thylakoids [28,34,35]). The slow decay of these absorption changes reflected reacidification by passage of protons from the lumen across the thylakoid membrane into the medium (half-
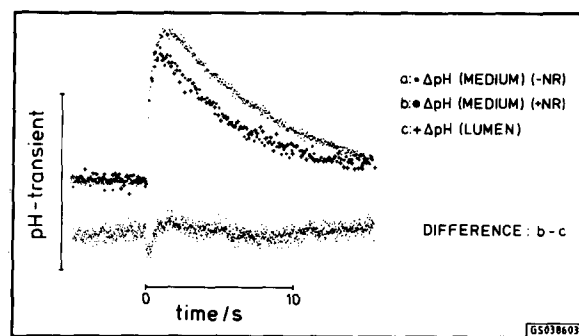

Fig. 2. Flash-induced absorption changes attributed to pH transients in the suspending medium and in the lumen of thylakoids, respectively. (a) Medium, indicator phenol red, no neutral red present, no bovine serum albumin, 559 nm. (b) Medium, indicator phenol red, neutral red added, no bovine serum albumin, 559 nm. (c) Lumen, indicator neutral red, bovine serum albumin present, 548 nm; bottom: difference of traces b and c. Freshly prepared chloroplasts were used, suspended at 10 µM chlorophyll in a medium with methyl viologen (10 µM) as electron acceptor for Photosystem I, $MgCl_2$ (2 mM), NaCl (30 mM) and at pH 7.0. Repetitive excitation at 30-s intervals. Traces a–c were obtained by subtracting transient signals measured with and without the respective pH-indicating dye. The bar at the ordinate represents a relative change of transmitted intensity: $\Delta I/I = 2 \cdot 10^{-3}$, except for trace c, which was upscaled by a factor of 3.0 (here, the bar reads: $\Delta I/I = 0.67 \cdot 10^{-3}$).

decay time, approx. 10 s). Similarly the rise of the absorption changes of neutral red reflected the acidification of the lumen (complex rise 100 µs to some ms, see Refs. 12–14) and their decay the leakage of protons from the lumen into the medium. It was puzzling that the apparent leakage time was shorter if measured in the lumen via neutral red (5 s, see Fig. 2c) than if measured in the medium via phenol red (10 s, see Fig. 2a). The reason for this discrepancy became obvious from the third trace (Fig. 2b). Here, we recorded the pH_{out}-indicating absorption changes of phenol red, in the presence of neutral red (15 µM). The extent of the absorption changes was diminished by 15%. This was attributable to the additional buffering capacity contributed by neutral red. The decay of the alkalinization was 2-fold accelerated from 10 s to 5 s half-decay time. This reflected the mild protonophoric action of neutral red (which, by the way, was also found by De Wolf et al. [24]). At equal concentration of this mild ionophore, neutral red, the decay times of the pH transients in the medium and in the lumen coincided. This was documented by the difference trace in the lower part of Fig. 2. The small deviation, from zero during the first 500 ms reflected the intrinsically different rise of the alkalization in the medium and of the acidification in the lumen.

The experiments which are documented in Fig. 2 led us to conclude the following. (a) Since the protonation–deprotonation of neutral red at interfaces occurs at submicroseconds [36], the velocity of proton transfer by neutral red is limited by the velocity of its passage across the membrane. The time for half relaxation of a pH difference across thylakoid membranes in the presence of neutral red (5 s) thus reflects its redistribution across the membrane in response to a small pH jump. (b) It was feasible to measure, in parallel and in both compartments, the time-course for the passage of protons across the membrane. As the kinetics from both types of measurement coincided, the fate of protons was tracked completely.

The latter result was particularly relevant for investigations aimed at the entry of protons into, at their passage across and at their reappearance at the other end of the ATP synthase or the open proton channel CF0. An example for this is illustrated in Fig. 3.
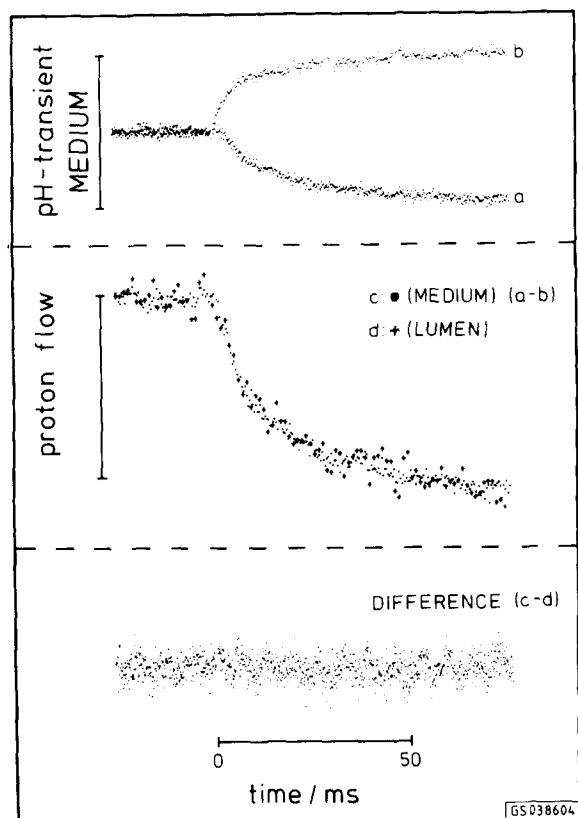
Fig. 3. pH-indicating absorption changes in thylakoids with approx. 30% of total CF1 extracted by EDTA-treatment. (a) Indicator phenol red, no DCCD present. (b) Indicator phenol red, DCCD present (25 μM, 10 min incubation). (c) Difference between traces a and b. (d) Indicator neutral red, difference between two traces with and without DCCD present (analogous to trace c). Thylakoids were suspended at 20 μM chlorophyll in a medium with hexacyanoferrate(III) (200 μM) as electron acceptor (at the given concentration it accepted from Photosystem I, only – see Fig. 1 in Ref. 28), NaCl (10 mM), no Mg^{2+} (to avoid restacking) and at pH 7.3. Repetitive excitation at 5-s intervals. The bars indicate relative changes of a transmitted intensity of 10^{-3}, except for trace d which was upscaled by a factor 4.4. (this factor was greater than the one used in Fig. 2, since fresh (Fig. 2) and EDTA-treated thylakoids (Fig. 3) differed in the extent of neutral red binding – for details, see Ref. 23). While traces a and b reflected the net pH change in the medium, traces c and d reflected the number of protons which passed from the lumen (d) across the thylakoid membrane into the medium (c).

The time-course of proton efflux from the lumen and of their influx into the suspending medium as mediated by open CF0-channels

The upper traces in Fig. 3 show the pH_{out}-indi-

cating absorption changes of phenol red in thylakoids where CF1 was extracted by low salt treatment. The degree of extraction, determined by immunoelectrophoresis, was appr. 30% of total CF1. In contrast to the experiments shown in Fig. 2, here hexacyanoferrate(III) was used as an electron acceptor. At the given low concentration (200 μM) it accepted electrons solely from Photosystem I (see Ref. 28). Therefore, flash excitation of thylakoids caused the uptake from the medium of one proton by Photosystem II only (1 H^+/PS II), while proton release into the lumen was caused by both photosystems (1 H^+/PS). The net result was an acidification of 1 H^+ per Photosystem I (see trace a in Fig. 3). On the other hand, after closure of the proton channel through CF0 by DCCD [26], one expected to see a relatively long-lasting alkalinization of the medium equivalent to the uptake of 1 H^+ per Photosystem II. This was indeed observed as shown by trace b in Fig. 3. Trace c shows the difference between traces a and b. It reflects the number of protons transported across the membrane as viewed from the medium via phenol red. Trace d also shows the number of transported protons, but viewed from the lumen via neutral red. It was obtained in an analogous way as trace c, but with neutral red (plus bovine serum albumin) instead of phenol red. Both traces rose with 10 ms half-rise time. At the given noise level the difference between traces c and d vanished (bottom trace in Fig. 3). Again proton tracking was complete.

Conclusions

In thylakoid suspensions with the medium buffered by bovine serum albumin which does not enter the lumen the flash-induced absorption changes of neutral red indicated transients of the surface pH at the lumenal side of the thylakoid membrane. This was previously established by quantitative studies on the influence of buffers and cations on these absorption changes and on the distribution of the dye between membrane and medium. These studies were carried out with freeze-thawed thylakoids [22,23]. In freshly prepared thylakoids, however, buffers and cations failed to act on these absorption changes. In this paper we showed that the extent and the complex

rise kinetics of these absorption changes were the same in both types of thylakoid. We concluded that neutral red indicated the lumenal surface pH also in freshly prepared thylakoids.

We measured the relaxation of a flash-induced pH-difference across the thylakoid membrane in two different ways: via phenol red in the medium and via neutral red in the lumen. We used thylakoids with proton-tight membranes (half-relaxation time of a pH transient, approx. 10 s) and membranes which were made leaky to protons by CF1 extraction through EDTA-treatment (half-relaxation time, 10 ms). In both types of membrane the respective relaxation time was independent of the measuring technique, viewed from the lumen via neutral red and viewed from the medium via phenol red. This demonstrated that proton passage across the membrane could be completely tracked.

In response to a flash-induced pH difference the redistribution of neutral red between the inner and the outer surface of the thylakoid membrane was rather slow (approx. 10 s). This guaranteed that bound neutral red straightforwardly acted as a pH indicator for small pH-transients at short time intervals. On the other hand it explained why the dye was less well suited for measurements at longer time scales.

Objections raised by De Wolf et al. [24] were discussed in the introduction.

Acknowledgements

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